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Supporting Online Material for

Kinetic Scaffolding Mediated by a Phospholipase C–β and G_q Signaling Complex

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MATERIALS AND METHODS

Materials. HisTrap HP and CM5 sensor chips (research grade) were obtained from GE Healthcare, DNAse I from Sigma, complete EDTA-free protease inhibitor from Roche, L-α-phosphatidylethanolamine (bovine heart and bovine liver), L-α-phosphatidylserine (porcine brain), and L-α-phosphatidylinositol-4,5-bisphosphate (porcine brain) from Avanti Lipids, and [γ-^{32}P]guanosine 5'-triphosphate and phosphatidylinositol 4,5-bisphosphate [inositol-2-^{3}H(N)] from PerkinElmer.

Crystallization, structure determination and refinement. Protein complexes were formed by combining 150 μM PLC-β3(ΔCT) with 165 μM Gαq(Δ34) in 20 mM HEPES, pH 8.0, 1 mM DTT, 2 % glycerol, 200 mM NaCl, 30 μM $AlCl_3$, 10 mM NaF, 2 mM $MgCl_2$, and 30 μM GDP and isolated by gel filtration chromatography over a Superdex S-200 HR10/30 column eluted with the same buffer. The protein complex was concentrated to 7.5 mg/ml. Crystals were obtained in hanging drops by vapor diffusion of a 1:1 (v/v) protein solution to well solution (7% PEG 6000, 100 mM HEPES, pH 6.0, 1 mM $CaCl_2$ and 100 mM $Mg(CH_3COO)_2$) at 18° C. Crystals of the space group C2 (a = 203.0 Å, b = 90.9 Å, c = 93.1 Å, α = 90°, β = 101.1°, γ = 90°) formed within 1 h and contained one Gαq(Δ34)•GDP•AlF_4•PLC-β3(ΔCT) complex in the asymmetric unit with a solvent content of approximately 53%. Cryo-protection was achieved by sequentially dipping the crystal in crystallization buffer containing 5%, 10%, and 20% (v/v) glycerol. A native dataset was collected on SER-CAT beamline 22-ID at the Advanced Photon Source at Argonne National Labs at 100 K. The data were indexed and processed using HKL2000. The CCP4 program Phaser[1] was used to locate a molecular replacement solution using search models obtained from the structures of PLC-β2 (PDB 2FJU)[2] and Gαq (PDB 2BCJ)[3]. The model was refined using CNS[4], Coot[5], and Refmac[6] and displayed using PyMOL[7]. Data collection and structure refinement statistics are shown in Table S1.

Mutagenesis and cloning. Mutations were introduced into the Hα1/Hα2 region and EF hand loop of PLC-β3 by insertion of DNA cassettes containing the desired mutations. QuikChange (Stratagene) mutagenesis was first utilized to introduce silent mutations into PLC-β3 resulting in addition of unique HindIII and XbaI sites at the 5′ and 3′ ends, respectively, of the 550 bp region encompassing the Hα1/Hα2 region of PLC-β3. Individual DNA cassettes for this 550 bp region in PLC-β3 were generated by PCR using a forward primer containing tandem 5′ KpnI and HindIII sites and a reverse primer containing a 3′ XbaI site. The PCR fragments were digested using KpnI and XbaI restriction enzymes and ligated into a pBS SK vector. Quikchange mutagenesis was used to introduce single point mutations into the Hα1/Hα2 region, and the mutated cassettes were digested with HindIII and XbaI restriction enzymes and ligated into the modified PLC-β3 plasmid.

A similar strategy was employed to generate mutations in the EF hand of PLC-β3, except that unique silent ClaI and BsrGI sites were added to the 5′ and 3′ ends, respectively, of the targeted 400 bp region. To generate EF hand DNA cassettes, PCR was used to amplify this 400 bp region with a forward primer containing a 5′ ClaI site and a reverse primer containing tandem 3′ BsrGI and BamHI sites. After digesting with ClaI and BamHI restriction enzymes and ligating into pBS SK vector, the DNA cassettes were mutated, digested with ClaI and BsrGI, and ligated into the modified PLC-β3 plasmid.

Single point mutations in the region preceding the C2 domain of PLC-β3 were generated using QuikChange mutagenesis according to the manufacturer's protocol. All mutations were confirmed by dideoxy sequencing. All baculovirus constructs for expression of PLC-β3 and PLC-β3 mutants included an N-terminal hexahistidine tag followed by a TEV protease site.

Chimeric constructs that incorporated Gαq-interacting regions of PLC-β3 into PLC-δ1 were generated using PLC-δ1 as the target vector. Briefly, primers and PCR were used to amplify the Hα1/Hα2 region of PLC-β3 to produce a 35 base pair 5' overhang complementary for the C-terminus of PLC-δ1 and a 35 base pair 3' overhang complementary for the vector. After gel extraction of the PCR product, a subsequent round of PCR was used to insert the Hα1/Hα2 region of PLC-β3 into the PLC-δ1 vector. All constructs were fully sequence verified.

Purification of PLC-β3 isozymes. Four liters of High-Five insect cells (2-3 x 10^6 cells/ml) were infected with recombinant baculovirus (30 ml/liter) and harvested 48 h later by centrifugation at 750 xg for 15 min in a JA10 rotor. All subsequent steps were at 4° C. The cell pellet was resuspended (30 ml/liter of culture) in lysis buffer (50 mM Hepes, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 15 mM imidazole, and protease inhibitors), and subjected to cell lysis using an EmulsiFlex-C5 (Avestin). The lysate was centrifuged at 25,900 xg for 10 min, the supernatants saved, and the pellets resuspended by dounce homogenization in 100 ml lysis buffer. The resuspended pellets were centrifuged at 25,900 xg for 10 min, all supernatants were combined, and the pellets were discarded. The supernatant was incubated with 5000 Units of DNAse I for 15 min prior to centrifugation at 100,000 xg for 60 min. The supernatant was collected and combined with lysis buffer containing 4 M NaCl to produce a final concentration of NaCl of 0.4 M prior to an additional centrifugation step at 100,000 xg for 30 min. This supernatant was collected, filtered through a 0.2 micron nitrocellulose filter, and loaded on a 1 ml HisTrap column at a flow rate of 1 ml/min. The HisTrap column was washed with 30 ml of load buffer (20 mM Hepes, pH 8.0, 400 mM NaCl, 10 mM β-mercaptoethanol, 15 mM imidazole, and protease inhibitors), and bound protein was eluted with load buffer containing 500 mM imidazole. EDTA (250 mM) was added to the eluate to produce a final concentration of EDTA of 1 mM, and the sample was subjected to chromatography over a 1.6 x 66 cm Sephacryl 200 column using a running buffer consisting of 20 mM Hepes, pH 8.0, 100 mM NaCl, 2 mM DTT, 10% glycerol, 5 mM $MgCl_2$, 50 µM GDP, 30 µM $AlCl_3$, 10 mM NaF, and protease inhibitors. The PLC-β3-containing fractions were pooled and treated with TEV protease overnight. NaCl (final concentration, 0.4 M) and imidazole (15 mM, final concentration) were added, and the sample was passed over a 1 ml HisTrap column. The flow-through was collected, concentrated, and passed over a 23 ml (1 x 25 cm) Superdex 200 column using a running buffer consisting of 20 mM Hepes, pH 8.0, 1 mM DTT, 2% glycerol, 200 mM NaCl, 5 mM $MgCl_2$,50 µM GDP, 30 µM $AlCl_3$, 10 mM NaF, and protease inhibitors. The PLC-β3-containing fractions were pooled, and the yield of purified protein was 5-10 mg per liter of infected cells.

Purification of PLC-δ1 containing the Hα1/Hα2 region of PLC-β3. Chimeric PLC-δ1 proteins were expressed in BL21 (DE3) *E.coli* cells from a pET-15b vector (Novagen) encoding an N-terminal hexahistidine tag followed by a TEV protease site. Transformed cells were grown in LB medium containing 0.1 mg/ml of ampicillin at 37°C to mid-log phase and induced with 100 µM isopropyl-β-d-thiogalactopyranoside at 22°C for 16 h. Cells were harvested at 1000 xg and resuspended in buffer containing 10 mM imidazole, 20 mM Tris, pH 8, 10% (v/v) glycerol, and 300 mM NaCl. Cell suspensions were lysed using an EmulsiFlex-C5 homogenizer (Avestin) and centrifuged at 25000 xg for 45 min at 4°C. The resulting supernatants were applied to a 5-ml HisTrap HP column (GE Healthcare) charged with Ni^{2+}, washed with 10 column volumes of buffer A (10 mM imidazole, 20 mM Tris, pH 8.0, 10% (v/v) glycerol, and 300 mM NaCl) and 5 column volumes of 5% buffer B (buffer A containing 1 M imidazole), and then eluted with a step gradient of 40% buffer B. Eluted chimeric proteins were dialyzed overnight in buffer A in the presence of TEV protease and subsequently were applied to a 1-ml His Trap HP column (GE Healthcare) to remove TEV. Proteins were then applied to a Superdex S200 16/70 size-exclusion column (GE Healthcare) equilibrated in 20 mM Tris, pH 8.0, 5% (v/v) glycerol, 300 mM NaCl, and 2 mM DTT. Fractions containing purified lipases were pooled and concentrated using a 30,000 MWCO Vivaspin 20 centrifugal filtering device (Vivascience) and stored at -80° after flash freezing in liquid nitrogen.

Purification of Gαq. A baculovirus expression vector was engineered for high level expression and purification of a chimeric Gα. The expressed chimeric protein included an N-terminal hexahistidine tag followed by residues 1-28 of rat Gαi1, a TEV protease site, and residues 35-359 of mouse Gαq (GαqΔ34). Four liters of High-Five insect cells (3 x 10^6 cells/ml) were infected with recombinant baculovirus (30 ml/liter) encoding the chimeric Gα and harvested 48 h later by centrifugation at 750 xg for 15 min. All subsequent steps were at 4°C. The cell pellet was resuspended (30 ml/liter of culture) in lysis buffer (50 mM Hepes, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 15 mM imidazole, 50 µM GDP, 30 µM $AlCl_3$, 10 mM NaF, and protease inhibitors), and subjected to cell lysis using an EmulsiFlex-C5 (Avestin). The lysate was centrifuged at 25,900 xg for 10 min, the supernatants saved, and the pellets resuspended by dounce homogenization in 100 ml lysis buffer. The resuspended pellets were centrifuged at 25,900 xg for 10 min, all supernatants were combined, and the pellets were discarded. The supernatant was incubated with 20,000

Units of DNAse I for 15 min prior to centrifugation at 100,000 xg for 60 min. The supernatant was collected and combined with lysis buffer containing 4 M NaCl to produce a final concentration of NaCl of 0.4 M prior to an additional centrifugation step at 100,000 xg for 30 min. This supernatant was collected, filtered through a 0.2 micron nitrocellulose filter, and loaded on a 1 ml HisTrap column at a flow rate of 1 ml/min. The HisTrap column was washed with 30 ml of load buffer (20 mM Hepes, pH 8.0, 400 mM NaCl, 10 mM β-mercaptoethanol, 15 mM imidazole, 50 μM GDP, 30 μM $AlCl_3$, 10 mM NaF, 10% glycerol, and protease inhibitors), and bound protein was eluted with 6 ml of load buffer containing 500 mM imidazole. EDTA (250 mM) was added to the eluate to produce a final concentration of 1 mM, and the sample was subjected to chromatography over a 1.6 x 66 cm Sephacryl 200 column using a running buffer consisting of 20 mM Hepes, pH 8.0, 100 mM NaCl, 2 mM DTT, 10% glycerol, and protease inhibitors. The Gαq-containing fractions were pooled and treated with TEV protease overnight. NaCl (final concentration, 0.4 M) and imidazole (15 mM, final concentration) were added, and the sample was passed over a 1 ml HisTrap column. The flow-through was collected, concentrated, and passed over a Superdex 200 HR10/30 column using a running buffer consisting of 20 mM Hepes, pH 8.0, 1 mM DTT, 2% glycerol, 200 mM NaCl, and protease inhibitors. The Gαq-containing fractions were pooled and concentrated using a VivaSpin-10 filter. The yield of purified protein was 1-2 mg per liter of infected cells.

Purification of lipidated $G\beta_1\gamma_2$, lipidated Gαq, and $P2Y_1$ receptor. Proteins were purified from membranes prepared from baculovirus-infected insect cells as we previously described[8].

Reconstitution of $P2Y_1$ receptor, Gαq,and $G\beta_1\gamma_2$ into proteoliposomes. Purified $P2Y_1$ receptor was reconstituted with purified Gαq and $G\beta_1\gamma_2$ into proteoliposomes as previously described[8]. Briefly, 15 pmol of purified human $P2Y_1$ receptor, 50 pmol Gαq and 75 pmol $G\beta_1\gamma_2$ were reconstituted by Sephadex G-50 chromatography into phospholipid vesicles containing L-α-phosphatidylserine from brain, L-α-phosphatidylethanolamine from liver, and cholesteryl hemisuccinate. For GTPase assays, proteoliposomes eluted at final concentrations equivalent to 238 μM phosphatidylethanol, 143 μM phosphatidylserine, and 13 μM cholesteryl hemisuccinate, and 3 μl was used in a 50 μl assay volume. For phospholipase assays proteoliposomes eluted at final concentrations equivalent to 238 μM phosphatidylethanolamine, 143 μM phosphatidylserine, 13 μM cholesteryl hemisuccinate, and 20 μM [^{3}H]PtdIns(4,5)P_2 and 10 μl was used in a 50 μl assay volume.

Quantification of phospholipase activity of purified PLC-β3 isozymes. Basal phospholipase activity was determined by combining phosphatidylethanolamine and [^{3}H]PtdIns(4,5)P_2 in a 10:1 molar ratio, and drying the lipids under N_2. The dried lipids were resuspended by sonication in buffer consisting of 20 mM Hepes, pH 7.4, 50 mM NaCl, 2 mM DTT, and 1 mg/ml fatty acid-free bovine serum albumin. Reactions were for 10 min at 30°C in a final buffer consisting of 20 mM Hepes, pH 7.4, 70 mM KCl, 10 μM free Ca^{++}, 2 mM DTT, 3 mM EGTA, 16 mM NaCl, 0.3 mg/ml fatty acid-free bovine serum albumin, 50 μM [^{3}H]PtdIns(4,5)P_2, and 6 nM PLC-β3.

$G\beta_1\gamma_2$-stimulated lipase activity was determined by combining phosphatidylethanolamine, phosphatidylserine, and [^{3}H]PtdIns(4,5)P_2 in a 1:4:1 molar ratio and drying the lipids under N_2. The dried lipids were resuspended by sonication in buffer consisting of 20 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT. Reactions were for 10 min at 25°C in a final buffer containing 40 mM Hepes, pH 7.4, 110 mM NaCl, 4.7 mM $MgCl_2$, 2 mM EGTA, 3 μM free Ca^{++}, 0.5 mg/ml fatty acid-free bovine serum albumin, and 25 μM [^{3}H]PtdIns(4,5)P_2, and PLC-β3 with or without 60 nM $G\beta_1\gamma_2$.

$P2Y_1$ receptor- and Gαq- stimulated lipase activity was determined with 10 μl of reconstituted vesicles (described above) in a 50 μl assay at 30°C. Assay times (1-10 min) were adjusted to maintain linearity of assays with various concentrations of either PLC-β3 or PLC-δ1.

Quantification of steady-state GTP hydrolysis. Steady-state GTPase activity of reconstituted proteoliposomes was determined[8] in the absence or presence of PLC-β3 with or without the $P2Y_1$ receptor agonist 2MeSADP (10 μM). Unless otherwise indicated, incubations (50 μl, final volume) were for 30 min at 30°C and contained 20 mM Hepes, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, and 2 μM [γ-^{32}P]GTP (~4500 cpm/pmol). Reactions were terminated by addition of 950 μl of a 4°C solution of 5% activated charcoal

in 20 mM H_3PO_4. Following centrifugation, liberated [^{32}P]Pi in the supernatant was quantified by liquid scintillation spectrometry.

Transfection of COS-7 cells and quantification of [^{3}H]Inositol phosphate accumulation. COS-7 cells were maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in an atmosphere of 90% air/10% CO_2. Cells were seeded in 12-well culture dishes at a density of ~65,000 cells per well 24 h prior to transfection. The indicated DNA vectors were transfected using FuGENE 6 (Roche Applied Science) transfection reagent according to the manufacturer's protocol. The total amount of transfected DNA was 800 ng and included empty vector to maintain an equal amount of DNA per well. The culture medium was changed approximately 24 h after transfection to inositol-free Dulbecco's modified Eagle's medium (ICN Biomedicals) containing 1 µCi/well *myo*-[2-^{3}H(N)]inositol (American Radiolabeled Chemicals). Metabolic labeling proceeded for 12-18 h. Accumulation of [^{3}H]-labeled inositol phosphates was quantified following 60 min incubation in the presence of the 10 mM LiCl to inhibit inositol phosphate phosphatases. Incubations were terminated by aspiration of the culture medium and subsequent addition of 50 mM formic acid followed by neutralization with 150 mM NH_4OH. [^{3}H]inositol phosphates were isolated and quantified using Dowex chromatography.

Surface plasmon resonance. Binding of phospholipase C isozymes to Gαq was monitored at 25 °C using surface plasmon resonance with a BIAcore3000 (Biacore) with flow rates of 20–25 µl/min, using Hepes-buffered saline with polysorbate 20 (supplemented with 5 mM $MgCl_2$). Similar immobilized protein surfaces were achieved for each flow cell on CM5 sensor chips (Biacore, GE Healthcare) by first activating each flow cell with an injection of a 1:1 mix of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and N-hydroxysuccinimide Each protein was captured on individual flow cells using injections of 0.2 mg/ml protein solutions followed by an injection of 1 mM ethanolamine to block remaining active surface groups as described previously. Surface densities of approximately 1000 -1500 response units were created for each experiment. Unless noted, sensorgrams were normalized to the signal achieved due to binding a control "blank" flow cell surface or a negative control surface prepared with aldolase. Increasing concentrations of Gαq, the analyte, were passed over the surface in running buffer containing 100 µM GDP or 100 µM GDP supplemented with 30 µM $AlCl_3$ and 20 mM NaF. Steady state Gαq binding was plotted versus Gαq concentration to generate saturation binding isotherms and Kd values were calculated in GraphPad Prism[9] using single site binding analysis. A surface prepared with RGS4 served to monitor productive Gαq binding; Gαi1 as analyte controlled for non-specific binding to the PLC surfaces.

Generation of Gαq surface maps. The sites of interaction between effectors and their heterotrimeric G proteins were mapped onto the surface of Gαq using PyMOL. These effector/Gα complexes included Gαs·adenylyl cyclase[10], Gαt·PDEγ[11], Gαq·GRK2[3], and Gαq•p63RhoGEF[12]. A multiple sequence alignment was used to identify the analogous residues in Gαq. Residues contacted by all four effectors were colored dark green and those contacted by three or fewer effectors were shown as lighter shades of green. Structures of seven RGS•Gα complexes[11,13-15] were used to highlight RGS contact sites on the surface of Gαq using PyMOL and include RGS1•Gαi1, RGS4•Gαi1, RGS8•Gαi3, RGS9•Gαt, RGS10•Gαi3, RGS16•Gαi1, and RGS16•Gαo. A multiple sequence alignment was used to determine the corresponding amino acids in Gαq. Gα residues interacting with all seven RGS proteins were colored dark purple whereas those residues that interact with fewer than seven RGS proteins were shown in lighter shades of purple.

Quantification of phototransduction. The N262A mutation was introduced into the *norpA* cDNA and subcloned between the NotI and XbaI sites of the pCNX vector[16]. The constructs were injected into *norpA^{P24}* embryos to generate the *norpA^{N262A}* transgenic flies. ERG recordings were performed as described previously[17]. Briefly, a glass microelectrode filled with Ringer's solution was inserted into small drops of electrode cream placed on the surfaces of the compound eye and the other microelectrode was placed on the thorax. A Newport light projector (model 765) was used for stimulation. The ERGs were amplified using a Warner electrometer IE-210 and recorded with a MacLab/4s A/D converter and the Chart v3.4/s program. The flies were dark adapted for 2 min before exposure to a 5 sec orange light stimulus. The maximal amplitude and the time required for 80% recovery after termination of each response was quantified.

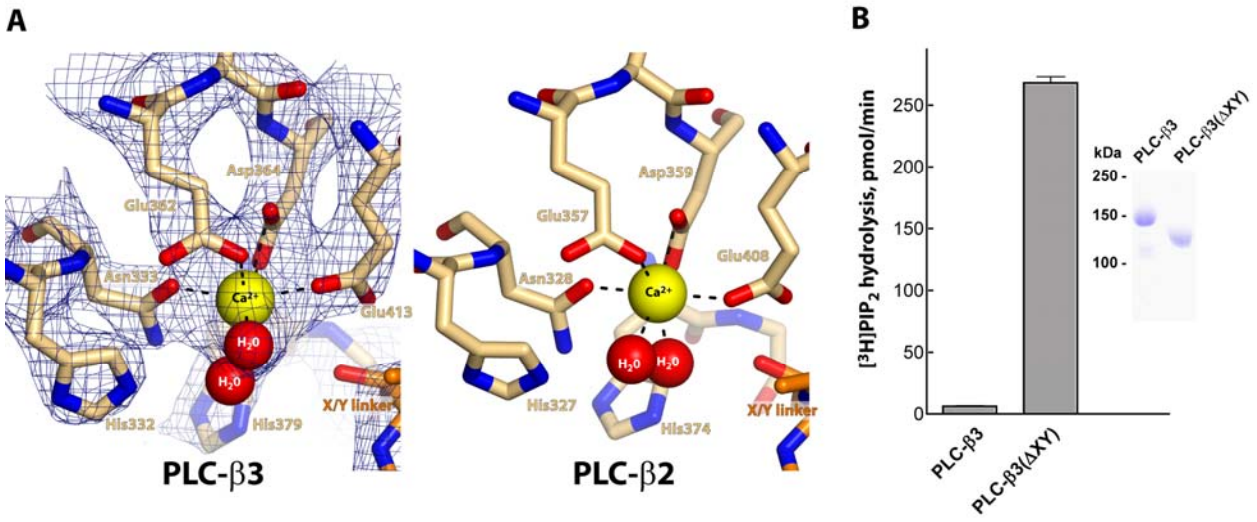

Figure S1. Activation of PLC-β involves removal of auto-inhibition mediated by the X/Y-linker. A, Comparison of the active site of PLC-β3 and PLC-β2. Dashed lines indicate interactions with the Ca^{2+} co-factor; blue mesh (left panel) represents electron density (2Fo - Fc map contoured at 1.2σ) for crystals of Gαq•PLC-β3. **B,** Removal of the X/Y-linker of PLC-β3 results in marked activation of the lipase. PLC-β3 and a deletion mutant of PLC-β3 lacking residues 472 through 585 (PLC-β3ΔXY) were purified as described in Supplementary Methods, and purity of the proteins was established by SDS-PAGE followed by staining with Coomassie brilliant blue (inset). One ng of each protein was incubated with mixed detergent-lipid vesicles containing [^{3}H]PtdIns(4,5)P_2, and formation of [^{3}H]Ins(1,4,5)P_3 was quantified as described in Supplementary Methods.

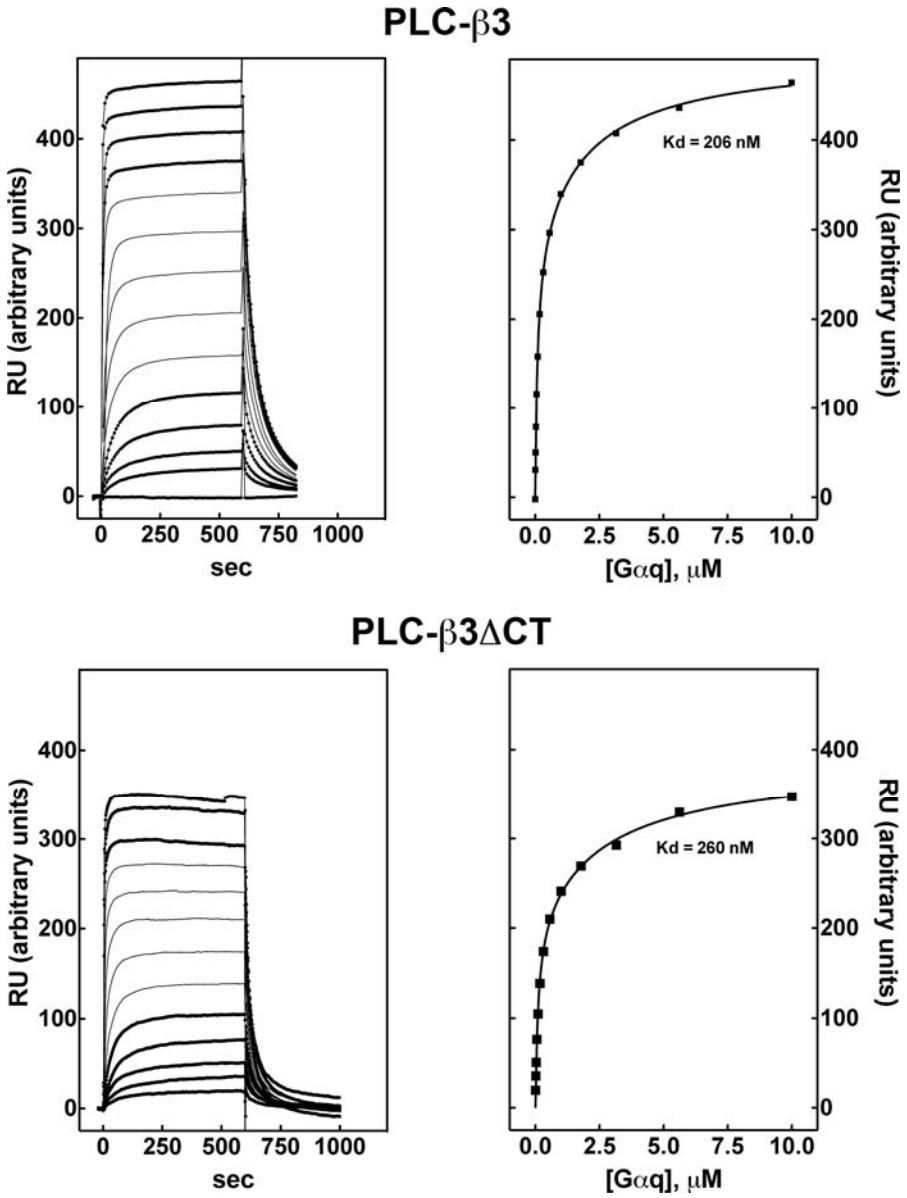

Figure S2. Determination of the binding affinity of PLC-β3 versus PLC-β3ΔCT for Gαq. Equivalent amounts of purified PLC-β3 or a construct of PLC-β3 truncated at residue 886 (PLC-β3ΔCT) were immobilized on a CM5 sensor chip (Biacore), and sensorgrams produced for increasing amounts of Gαq injected in the presence of AlF_4^- (10 mM NaF, 30 μM $AlCl_3$) as described in Supplementary Methods. These data were converted to saturation binding isotherms using Prism software (GraphPad, San Diego, CA).

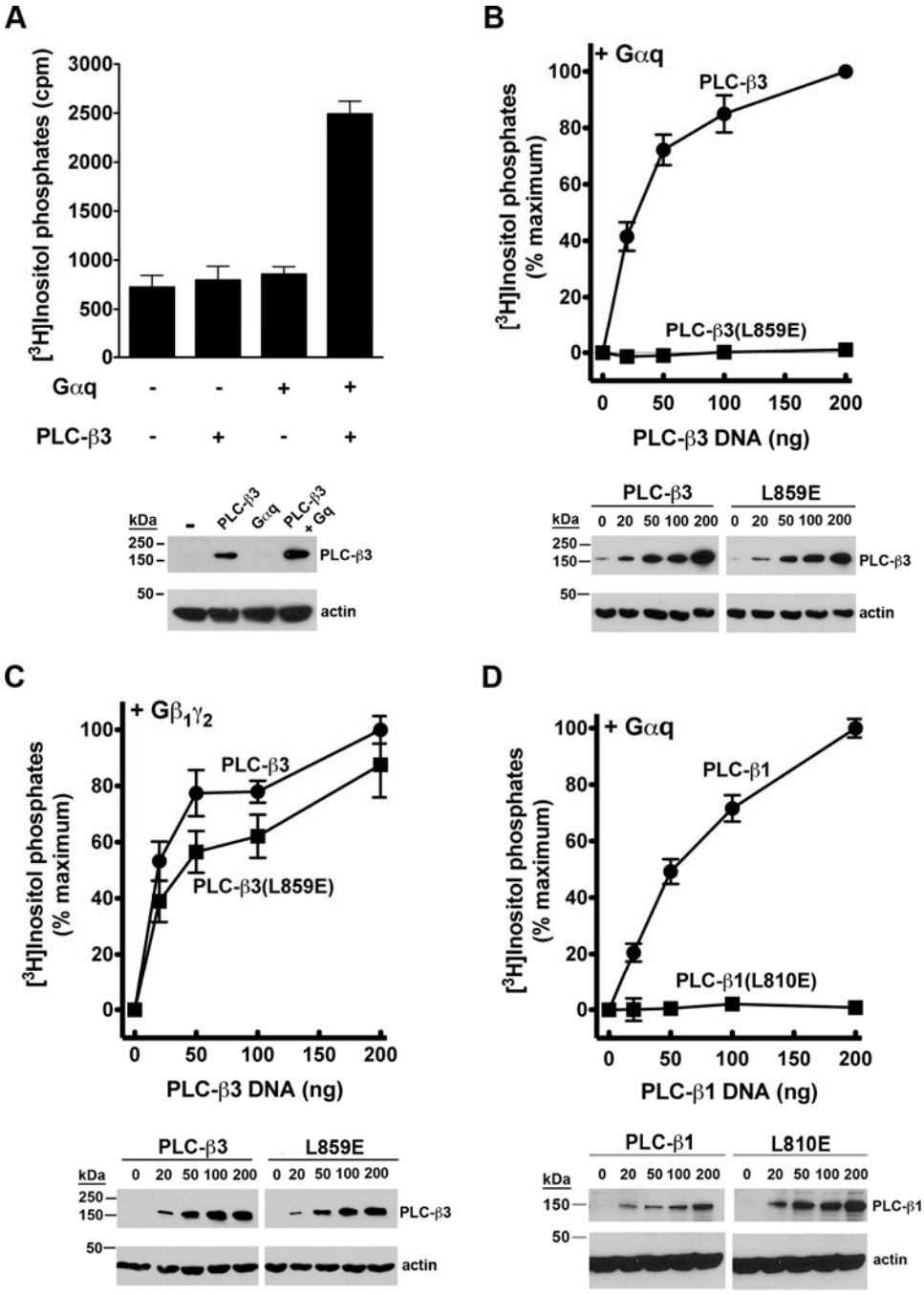

Figure S3. Loss of Gαq-mediated activation of PLC-β mutated in conserved leucine of the Hα1/Hα2 region. A, Gαq- and PLC-β3-dependent [^{3}H]inositol phosphate accumulation in COS-7 cells. COS-7 cells were transfected with 200 ng expression vector for Gαq, 100 ng of expression vector for PLC-β3, or these expression vectors in combination. [^{3}H]Inositol phosphate accumulation was quantified as detailed in Supplementary Methods. **B,** Mutation of L859 abrogates Gαq- promoted activation of PLC-β3. Gαq-dependent activation of PLC-β3 versus PLC-β3(L859E) was compared by quantification of [^{3}H]inositol phosphate accumulation after transfection of COS-7 cells with the indicated amounts of expression vectors for PLC-β3 or PLC-β3(L859E) and 200 ng Gαq. Data are plotted as percent of the maximal response of PLC-β3 to Gαq. Relative expression of PLC-β3 and PLC-β3(L859E) was quantified under each transfection condition using a PLC-β3-specific antibody, and actin immunoblots were utilized as loading controls for the SDS-PAGE

gels. **C,** Mutation of L859 does not abrogate Gβγ-promoted activation of PLC-β3. Gβγ-dependent activation of PLC-β3 versus PLC-β3(L859E) was compared by quantification of [^{3}H]inositol phosphate accumulation after transfection of COS-7 cells with the indicated amounts of expression vectors for PLC-β3 or PLC-β3(L859E) and 300 ng of $G\beta_1$ and 300 ng of $G\gamma_2$. Data are plotted as percent of the maximal response of PLC-β3 to $G\beta_1\gamma_2$. Relative expression of PLC-β3 and PLC-β3(L859E) was quantified under each transfection condition using a PLC-β3-specific antibody, and actin immunoblots were utilized as loading controls for the SDS-PAGE gels. **D,** Mutation of the conserved leucine in the Hα1/Hα2 region of PLC-β1 abrogates Gαq-stimulated inositol phosphate accumulation. Gαq-dependent activation of PLC-β1 versus PLC-β1(L810E) was compared by quantification of [^{3}H]inositol phosphate accumulation after transfection of COS-7 cells with the indicated amounts of expression vectors for PLC-β1 or PLC-β1(L810E) and 200 ng Gαq. Data are plotted as percent of the maximal response of PLC-β1 to Gαq. Relative expression of PLC-β1 and PLC-β1(L810E) was quantified under each transfection condition using a PLC-β1-specfic antibody, and actin immunoblots were utilized as loading controls for the SDS-PAGE gels.

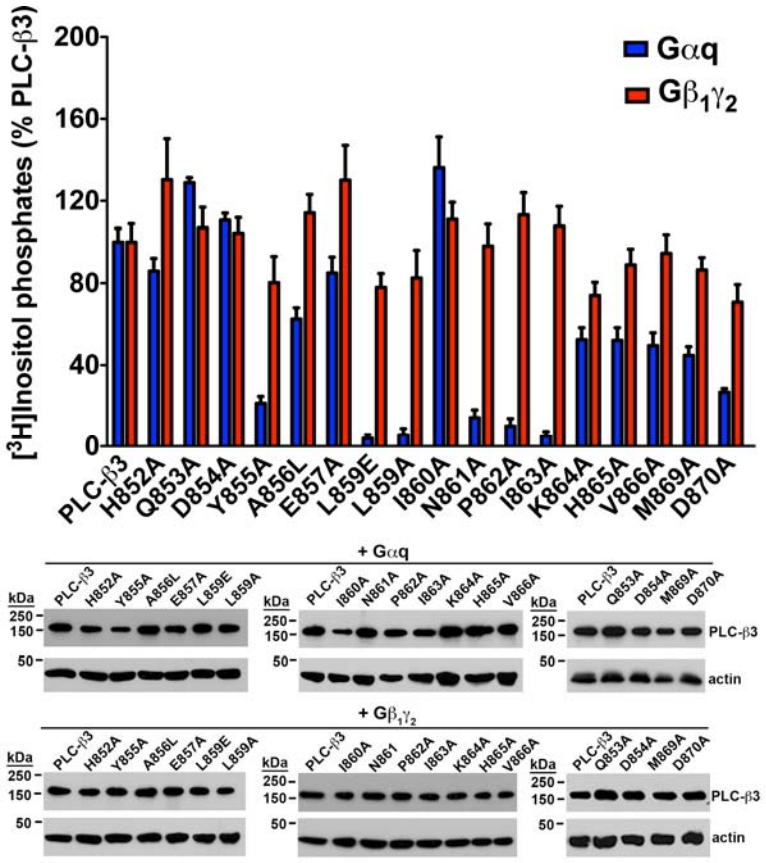

Figure S4. Mutational analysis of the Hα1/Hα2 region of PLC-β3. Gαq- and $G\beta_1\gamma_2$-dependent activation of PLC-β3 versus PLC-β3 mutants was determined by quantification of [^{3}H]inositol phosphate accumulation after transfection of COS-7 cells with 50 ng of expression vector for the indicated PLC-β3 mutants with vectors for either Gαq (200 ng) or $G\beta_1$ (300 ng) + $G\gamma_2$ (300 ng). Data for each PLC-β3 mutant are plotted as percent of the response of PLC-β3 to Gαq or $G\beta_1\gamma_2$. Relative expression of PLC-β3 and PLC-β3 mutants was quantified under each transfection condition using a PLC-β3-specfic antibody.

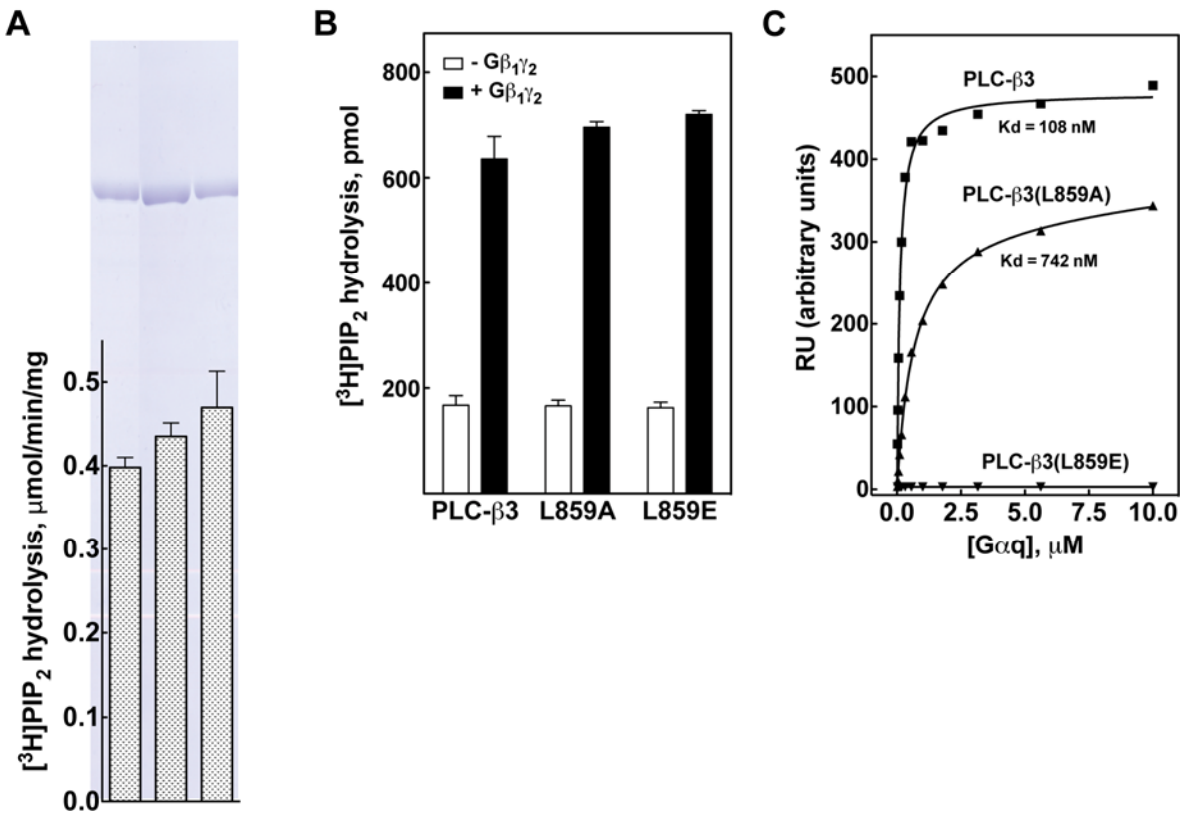

Figure S5. Quantification of activities of purified PLC-β3 mutated in a conserved leucine of the Hα1/Hα2 region. A, PLC-β3, PLC-β3(L859A), and PLC-β3(L859E) were purified to homogeneity after expression from recombinant baculoviruses as described in Supplementary Methods. A Coomassie-stained SDS-PAGE gel of these proteins is presented. Lipase activity of each purified PLC-β3 was quantified using mixed detergent-phospholipid micelles containing [^{3}H]PtdIns(4,5)P_2 as described in Supplementary Methods. **B,** Purified PLC-β3, PLC-β3(L859A), and PLC-β3(L859E) were incubated with [^{3}H]PtdIns(4,5)P_2-containing vesicles in the absence or presence of purified G$\beta_1\gamma_2$ (final concentration, 60 nM), and [^{3}H]Ins(1,4,5)P_3 was quantified as described in Supplementary Methods. **C,** Binding affinities of purified mutants of PLC-β3 were determined by surface plasmon resonance analyses. Equivalent amounts of PLC-β3, PLC-β3(L859A), or PLC-β3(L859E) were immobilized on a CM5 sensor chip (Biacore), and sensorgrams produced for increasing amounts of Gαq injected in the presence of AlF_4^- (10 mM NaF, 30 μM $AlCl_3$) as described in Supplementary Methods. These data were converted to saturation binding isotherms using Prism software (GraphPad, San Diego, CA).

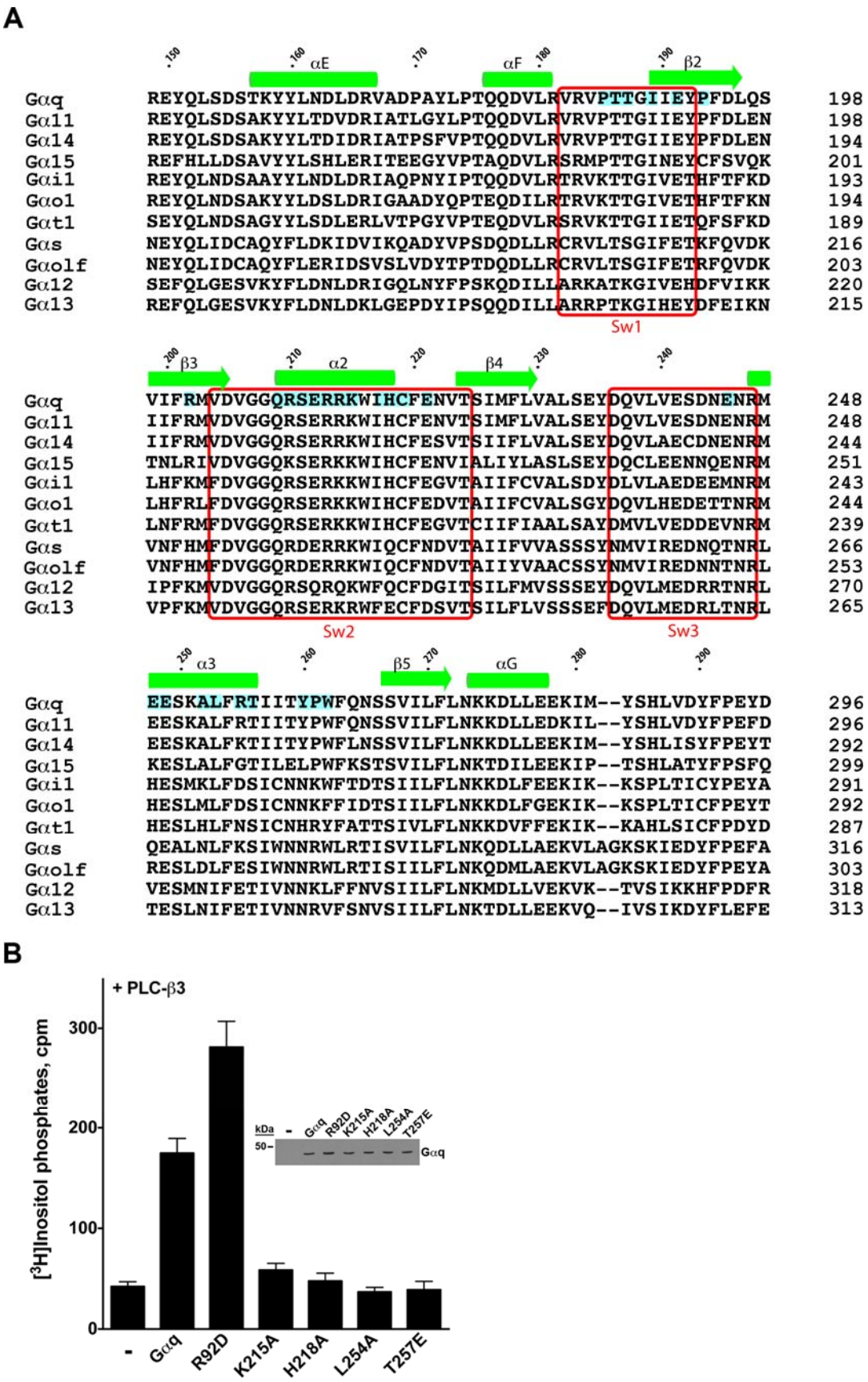

Figure S6. Sequence alignment of PLC-β3-interacting residues and mutational analysis of Gαq. A, The three conserved switch regions (red box) and the α3/β5 sequences of four

subclasses of heterotrimeric G proteins are presented in a multiple sequence alignment. PLC-β3-interacting residues of Gαq are highlighted in cyan. Secondary structure is indicated by cylinders (α-helices) and arrows (β-strands). **B,** Certain of the PLC-β3-interacting residues identified in the crystal structure of the Gαq•PLC-β3 interface were mutated to alanine, and the capacity of these mutants were tested after transfection of COS-7 cells with 100 ng of expression vector for Gαq, 200 ng of expression vector for PLC-β3, or these expression vectors in combination. [^{3}H]Inositol phosphate accumulation was quantified as detailed in Supplementary Methods. Relative level of expression of each Gαq was quantified using a Gαq-specific antibody.

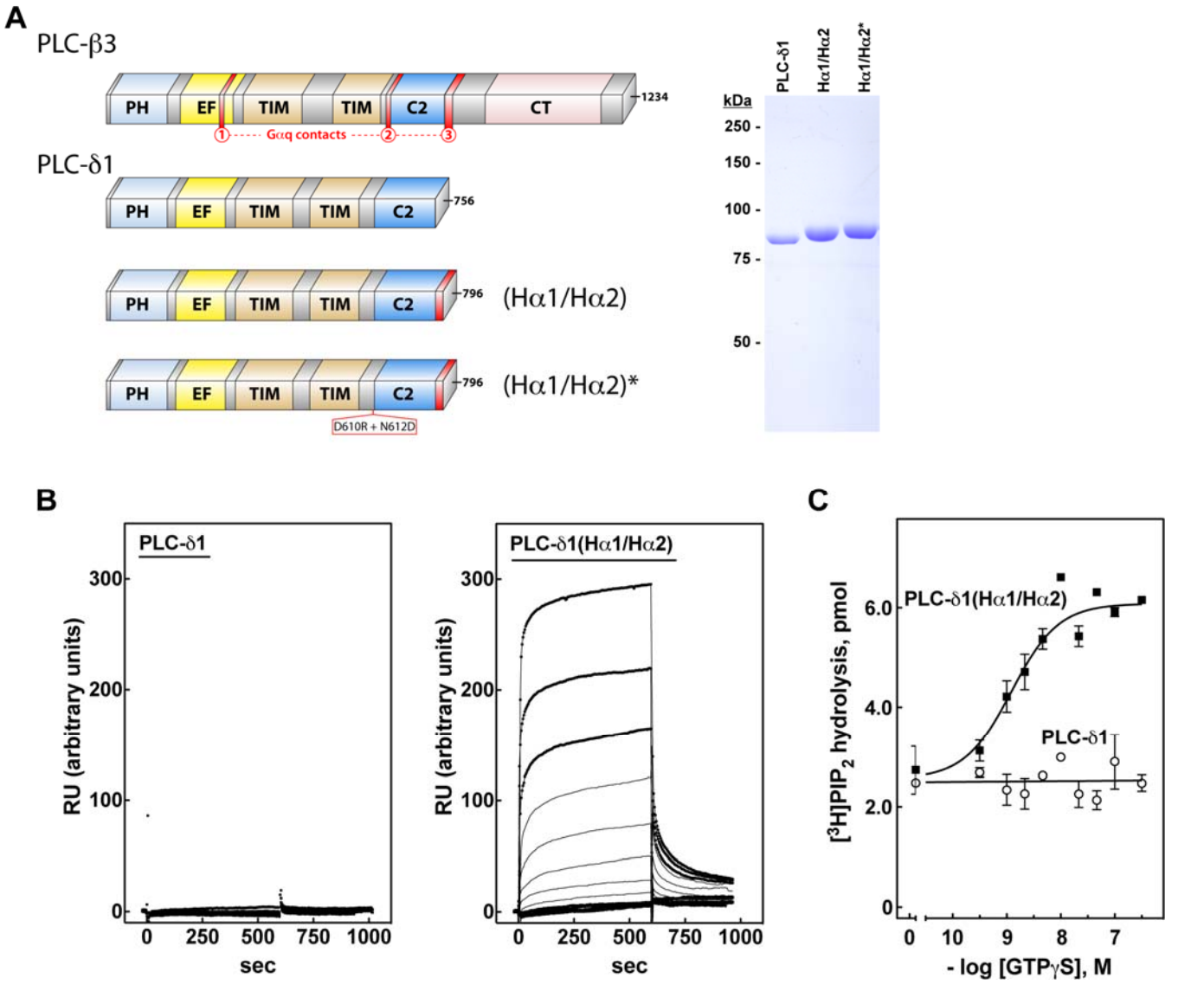

Figure S7. The Hα1/Hα2 region of PLC-β3 confers to PLC-δ1 a sensitivity to bind and be activated by Gαq. A, The domain architectures of PLC-β3 and PLC-δ1 are drawn to scale, and the N-terminal PH domain, a series of four EF-hands, the catalytic TIM barrel, and the C2 domain are indicated. PLC-β3 also contains a C terminal (CT) domain, and the three regions of PLC-β3 that are important for interaction with Gαq are indicated in red. Two chimeric forms of PLC-δ1 were generated that incorporate either the Hα1/Hα2 region [PLC-δ1(Hα1/Hα2)] or both the Hα1/Hα2 region and mutations (D610R and N612D) to PLC-β-specific residues in the region between the TIM barrel and C2 domain [PLC-δ1(Hα1/Hα2)*]. A Coomassie-stained SDS-PAGE gel of these proteins is presented. **B,** Equivalent amounts of purified PLC-δ1 or PLC-δ1(Hα1/Hα2) were covalently immobilized on a CM5 sensor chip, and sensorgrams produced by injecting increasing concentrations of Gαq in flow buffer containing 10 mM NaF and 30 μM $AlCl_3$. **C,** The concentration dependence of GTPγS for stimulation of lipase activity was quantified for PLC-δ1 (○) and PLC-δ1(Hα1/Hα2) (■) using [^{3}H]PtdIns(4,5)P_2-containing phospholipid vesicles reconstituted with purified $P2Y_1$ receptor, Gαq, and $Gβ_1γ_2$. Assays were in the presence of 30 nM PLC isozyme, 1 μM 2MeSADP, and the indicated concentrations of GTPγS.

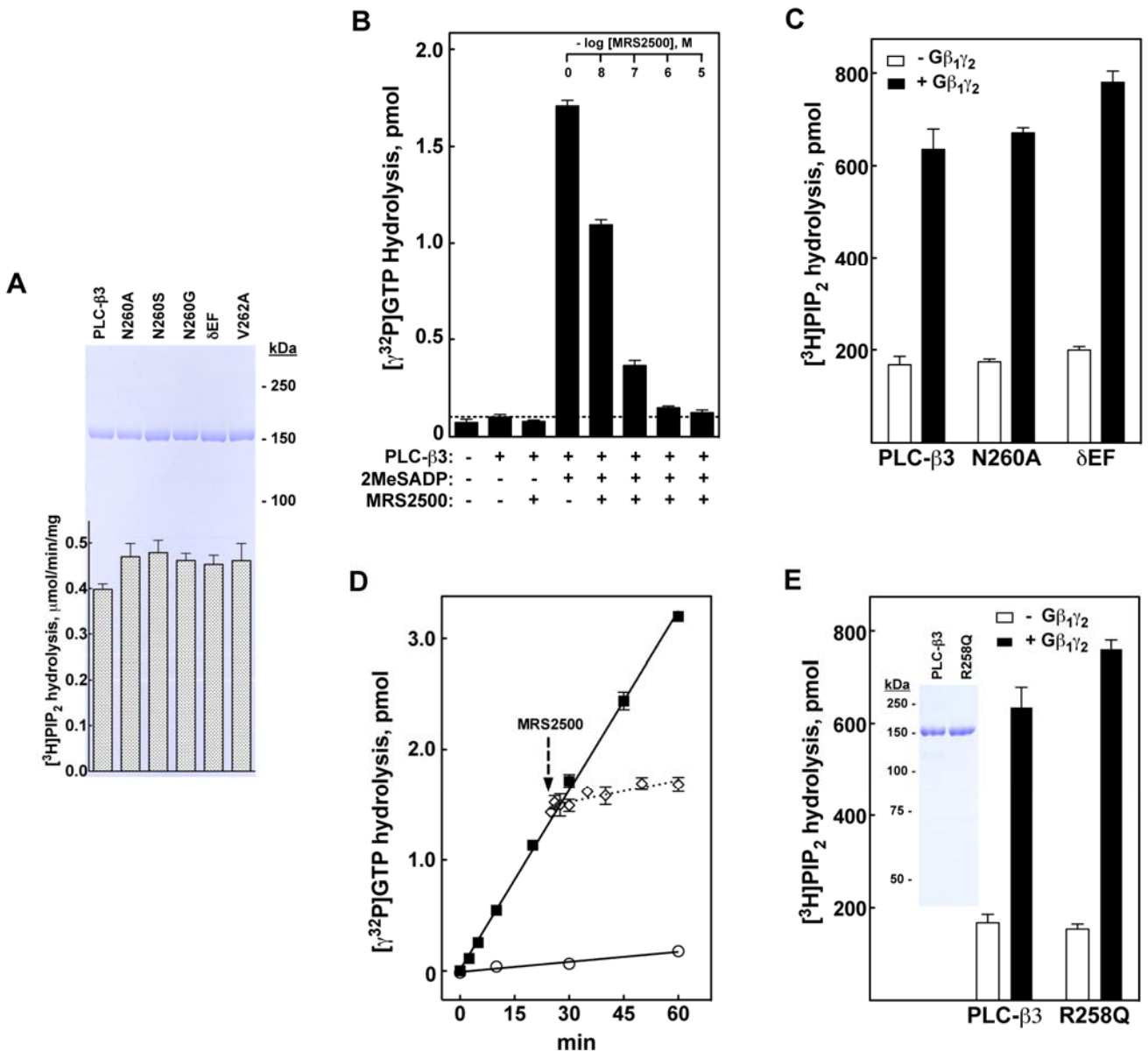

Figure S8. Quantification of GTP hydrolysis by Gαq in the presence of PLC-β3. A, PLC-β3, PLC-β3(N260A), PLC-β3(N260G), PLC-β3(N260S), PLC-β3(δEF), and PLC-β3(V262A) were purified to homogeneity after expression from recombinant baculoviruses as described in Supplementary Methods. A Coomassie-stained SDS-PAGE gel of these proteins is presented. Lipase activity of each purified PLC-β3 was quantified at a concentration of 3 nM using mixed detergent-phospholipid micelles containing [^{3}H]PtdIns(4,5)P_2 as described in Supplementary Methods. **B,** Steady-state GTP hydrolysis was quantified with phospholipid vesicles reconstituted with purified P2Y$_1$ receptor, Gαq, and Gβ$_1$γ$_2$ as described in Supplementary Methods. Assays were in the absence (indicated by -) or the presence (indicated by +) of 300 nM 2MeSADP and/or 300 nM PLC-β3 and/or the indicated concentrations of the P2Y$_1$ receptor antagonist MRS2500. **C,** Purified PLC-β3, PLC-β3(N260A), and PLC-β3(δEF) were incubated with [^{3}H]PtdIns(4,5)P_2-containing vesicles in the absence or presence of purified Gβ$_1$γ$_2$ (final concentration, 60 nM) and [^{3}H]Ins(1,4,5)P_3 was quantified as described in Supplementary Methods. **D,** Rapid termination of agonist + PLC-β3-stimulated GTP hydrolysis was assessed after addition of a P2Y$_1$ receptor antagonist. GTP hydrolysis was quantified at various times in the presence of 100 nM PLC-β3, phospholipid vesicles reconstituted with purified P2Y$_1$ receptor, Gαq, and

$G\beta_1\gamma_2$, and in the absence (○) or presence of 300 nM 2MeSADP (■). The receptor antagonist MRS2500 (10 μM) was added (arrow) after 25 min, and the incubation continued for an additional 35 min (◇). **E,** PLC-β3 and PLC-β3(R258Q) were purified to homogeneity after expression from recombinant baculoviruses as described in Supplementary Methods. A Coomassie-stained SDS-PAGE gel of these proteins is presented (inset). Purified PLC-β3 and PLC-β3(R258Q) were incubated with [^{3}H]PtdIns(4,5)P_2-containing vesicles in the absence or presence of purified $G\beta_1\gamma_2$ (final concentration,100 nM), and [^{3}H]Ins(1,4,5)P_3 was quantified as described in Supplementary Methods.

Table S1. Crystallization statistics for PLC-β3•Gαq.

Data Collection	
Space group	C2
Cell dimensions	
a, b, c (Å)	203.0, 90.9, 93.1
α, β, γ (°)	90, 101.2, 90
Resolution (Å)	50-2.70 (2.77-2.70)
R_{sym} [a]	7.0 (28.2)
$\langle I/\sigma \rangle$ [b]	20.3 (4.3)
Completeness (%)	91.7 (63.8)
Redundancy	4.9 (4.4)
Wavelength (Å)	1.000
Refinement	
Resolution (Å)	50-2.70
No. reflections (work/test)	37755/2152
R_{work} [c] / R_{free} [d]	20.4/27.5
No. atoms	
protein	8717
ligand	33
ion	2
water	227
B-factors (Å^2)	
protein	76.5
ligand	49.9
water	68.4
R.m.s deviations	
bond lengths (Å)	0.015
bond angles (°)	1.570
Ramachandran plot (%)	
favored	86.1%
allowed	13.9
disallowed	0

Numbers in parenthesis reflect highest resolution shell.

[a] $R_{sym} = 100 \times \sum | I - \langle I \rangle | / \sum I$, where I is the integrated intensity of a measured reflection.

[b] $\langle I/\sigma \rangle$ = mean signal to noise, where I is the integrated intensity of a measured reflection, and σ is the estimated error in the measurement.

[c] $R_{work} = \sum | Fp - Fp(calc) | / \sum Fp$, where Fp and Fp(calc) are the observed and calculated structure factor amplitudes.

[d] R_{free} = calculated as defined by R_{work} using 5% of the test set of reflections.

Table S2. EC_{50} values of purified PLC-β3 mutants for stimulation of GTP hydrolysis by Gαq.

	EC_{50} (nM)	n
PLC-β3	3.2 +/- 0.8	8
PLC-β3(V262A)	4.3 +/- 0.5	4
PLC-β3(N260A)	5.4 +/- 0.8	4
PLC-β3(N260G)	5.1 +/- 0.7	3
PLC-β3(N260S)	4.4 +/- 1.2	3
PLC-β3(δEF)	18.2 +/- 2.5	3
PLC-β3(L859A)	29.3 +/- 12.0	5
PLC-β3(L859E)	n.d.	3
PLC-β3(I860A)	1.3 +/- 0.6	2

The concentration dependence of purified PLC-β3 mutants for stimulation of GTP hydrolysis by Gαq was quantified with phospholipid vesicles reconstituted with purified $P2Y_1$ receptor and heterotrimeric Gq as is illustrated in Fig. 5A. EC_{50} values were determined using Prism software (GraphPad, San Diego, CA), and the values presented are the mean ± SEM of 3-5 separate experiments. n.d. – not detected.

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